METHOD DEVELOPMENT AND VALIDATION FOR ANTI DIABETIC DRUGS BY RP-HPLC

A dissertation submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

CHENNAI- 600 032

In partial fulfillment of the requirements for the award of degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

SUBMITTED

BY

YAMUNA PRADEEPA J

(Reg. No. 261330961)

Under the guidance of

Mr.S.Justin Jayaraj, M.Pharm.,

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

EDAYATHANGUDY.G.S PILLAY COLLEGE OF PHARMACY

NAGAPATTINAM-611002

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APRIL 2015

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CERTIFICATE

This is to certify that the dissertation entitled **"METHOD DEVELOPMENT AND VALIDATION FOR ANTI DIABETIC DRUGS BY RP-HPLC"** submitted by YAMUNA PRADEEPA J(Reg No: **261330961**) in partial fulfillment for the award of degree of Master of Pharmacy to the Tamilnadu Dr. M.G.R Medical University, Chennai is an independent bonafide work of the candidate carried out under my guidance in the Department of Pharmaceutical Analysis, Edayathangudy.G.S Pillay College of Pharmacy during the academic year 2012-2013.

Place: Nagapattinam

Date:

Mr.S.Justin Jayaraj, M.Pharm.,

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Place: Nagapattinam

Date:

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INTRODUCTION

Analytical chemistry may be defined as the science and art of determining the composition of materials in terms of the elements of composition contained. Pharmaceutical analysis is a bench of science that deals with the analytical procedures used to determine the purity, safety and quality of drugs and chemicals. It contains procedures to determine the identity , strength , quality and purity of new compounds . It also involves procedures for separating, identifying, and determining the relative amount of the components in sample of matter.

Quality assurance plays a key role in finding the safety and efficiency of medicines. It has highly specific and sensitive analytical methods for the design , development, standardization and quality control of medicinal products . They are equally important for the pharmacokinetics and drug metabolism studies, both which are important for the assessment of bioavailability and clinical response.

Modern physical method of analysis are extremely sensitive even for small amount of samples of materials .It can be rapidly applied and can readily amenable to automation. So it is widely used in the product development and in the control of manufacture , formulation and also in monitoring the use of drugs and medicines.

The term pharmaceutical analysis includes both quantitave and qualitative analysis of Drugs and pharmaceutical substances starting from bulk drugs to finished dosage forms .So it is used as a diagnostic aids in the modern practice of medicine by the analysis of chemical constituents in the human body which may alter during the disease state .

If the quality of drug product is questioned by a physician , the pharmacist is responsible for taking necessary steps to determine if indeed the product is defective . This may be accomplished by contacting with drug manufacturer about the problem involving the product , analyzing the preparation in the laboratory , borrowing needed equipment from a

clinical laboratory if necessary , sending the sample to a private laboratory for analysis , or a combination of all these steps .In any case ,however, it remains the responsibility of analyst to solve problems relating to drug quality **.**

The term 'quality' as applied to a drug product has been defined as the sum of all factors which contributes directly or indirectly to the safety , effectiveness and reliability of the product.

Significance of quality control:

The pharmaceutical industry continues as a vital segment of the health care cycle in conducting research and manufacturing products which are life maintaining and life restoring.

Modern medicines for human use are required to meet exacting standards which are Related to their quality , safety and efficacy .The evaluation of safety , efficacy and their maintenance in practice is dependent up on the existence of adequate methods for the quality control of product.

Quantitative analysis reveals the chemical identity of species in the sample . Quantitative analysis establishes the relative amount of one or more of these species or analyte in numerical terms.

Analytical Techniques

The efficacy and safety of a medicinal product can only be assured by analytical monitoring of its quality. Therefore, the overall purity of a medicine must be assessed throughout its storage, distribution and use.

The objective can possibly be achieved if the specification to be applied are based on validated procedure , which can demonstrate the relationship in quality between the substance under examination and that initially subjected to pharmaceutical, toxicological and pharmacological evaluation.

These are the following techniques employed for estimation of different components in formulations**.**

Optical methods

Some of the optical methods are

- X-ray spectroscopy
- UV-Visible spectroscopy
- Infrared spectroscopy
- Atomic absorption spectroscopy
- Flame photometry
- Nuclear magnetic resonance spectroscopy
- Nephlo-turbidimetry
- Electron spin spectroscopy

Electro analytical methods:

Some of the electro analytical methods are

- Amperometry
- Voltametry
- Potentiometry
- Conductometry

Separation methods/chromatography

Some of chromatographic methods are

- Gas-liquid chromatography
- Gas-solid chromatography
- Liquid-liquid chromatography
- Liquid-solid chromatography
- Thin-layer chromatography
- Paper chromatography
- Gel permeation chromatography

One of the major decisions to be made by an analyst is the choice of the most effective procedure for a given analysis , for this he must be familiar with the practical details, the theoretical principles and also that he must be conversant with the condition under which each method is reliable , aware of possible interferences which may arise and capable of demising means of circumventing such problems .

The instrumental separative techniques are divided into two categories.

1. Chromatography
2. Electrophoresis
3. Mass spectroscopy

Chromatography

Chromatography is a technique by which a mixture is separated into its components as a result of the relative ability of each component to be eluted along or through the stationary phase by mobile phase .The sample is placed on edge of the stationary phase (a solid or liquid) and a mobile phase is allowed to flow over the stationary phase to sweep the sample along the length of the stationary phase .Component which are strongly adsorbed to the stationary phase are swept less rapidly along the length of the stationary phase than those components that are less strongly adsorbed to stationary phase .

The word chromatography is derived from the Greek letters *chromos* meaning colour and the *graphy* means colour writing. The initial use of the terms is attributed to Tswett, who separated colour bands of plant pigments on a chromatography column that consist of an adsorbent powder that was washed with a liquid solvent termed as mobile phase . This is carried down the length of the tube that contains an immobile solid or liquid phase i.e. stationary phase.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The technique of high performance liquid chromatography (HPLC) was developed in the later 1960s and early 1970s from knowledge of the theoretical principles that already had been established for earlier chromatographic techniques in particular for column chromatography. The technique is based on the same modes of separation as classical column chromatography. I.e. adsorption, partition (including reverse phase partition), ion exchange and gel permeation. HPLC differ from column chromatography in that mobile phase is pumped through the packed column under high pressure. The principal advantages of HPLC compared to classical (gravity feed) column chromatography are improved resolution of the separated substances, faster separation times and the increased accuracy, precision and sensitivity with which the separated substances may be quantified**.**

Basic principle of HPLC

High performance liquid chromatography (HPLC) is a separation technique utilizing differences in distribution of compounds in phases called stationary phase and mobile phase. The stationary phase designates a thin layer created on the surface of fine particles and the mobile phase designates the liquid flowing over the particles. Under a certain dynamic solution, each component in a sample has different distribution equilibrium depending on the solubility in the phases and the molecular size.

As a result, the component move at different speeds over the stationary phase and there by separated by each other. The column is a stainless steel (or resin) tube, which is packed with spherical solid particles. Mobile phase constantly fed into the column inlet at a constant rate by a liquid pump. Sample is injected from sample injector, located near the column inlet. The injected sample enters the column with mobile phase and the components in these samples migrate through it, passing between the stationary phase and mobile phase.

Compound move in the column only it is in the mobile phase and therefore migrate faster through the column while compounds that tend to be distributed in the stationary phase migrate slower .In this way, component is separated on the column and sequentially elutes from the outlet. A detector connected to the outlet of the column detects each compound eluting from the column.

The recorder starts at the time when sample is injected and monitors the separation process and a graph is obtained. This graph is called chromatogram. The time that is required for a compound to elute (called retention time) and the relationship between the compound concentration (amount) and peak area depends on the characteristics of the compound.

Selectivity of HPLC

Most of the drugs can be analysed by HPLC method because of several advantages.

- Speed (analysis can be accomplished in 20 minutes or less).
- Greater sensitivity (various detectors can be employed).
- Improved resolution (wide variety of stationary phase).
- Reliable columns (wide variety of stationary phase).
- Ideal for substances of low volatility.
- Easy sample recovery, handling and maintenance.
- Easy programming of the numerous functions in each module.
- Time programmable operation sequence, such as initiating operation of detector lamp and pump to obtain stable baseline and equilibrated column before the work day begins.
- Excellent reproducibility of retention time.

Different Modes of Separation of HPLC

- Normal phase mode
- Reverse phase mode
- Reverse phase ion pair chromatography
- Ion exchange chromatography
- Affinity chromatography
- Size exclusion chromatography (gel permeation and gel filtration chromatography)

Instrumentation for HPLC

- A solvent reservoir for the mobile phase to be delivered to column over a wide range of flow rates and pressure. A degasser is needed to remove dissolved air and other gases from the solvents
- A pump to deliver the mobile phase to the column. The pumping system must be pulse free. A pump should be able to operate to at least 100 atm(1500 psm), pressure suited to less expensive chromatography. However, 400 atm (600 psi) is a more desirable pressure limit. For many analytical columns only moderate flow rate of 0.5 to 2 ml per minute needed to be generated.
- Sampling valves or loops are used to inject the sample in the flowing mobile phase just at the head of the separation column.
- At the head of the separation column, there may be a guard column or a inline filter to prevent contamination of the main column by small particulates.
- The separation column contains the packing needed to accomplish the desired HPLC separation. These may be silica's for adsorption chromatography, bonded phases for liquid-liquid chromatography, ion exchange functional groups bonded to stationary support for ion exchange chromatography, gels of specific porosity for exclusion chromatography, or some unique packing for a particular separation method.

- Most column lengths ranges from 10 – 30 cm, short, fast columns are 3 – 8cm long with an internal diameter of 4 -5 mm. Particle diameter lie in the range 3 -5 µm, occasionally up to 10 µm or higher for preparative chromatography.
- A detector with some type of data handling device, completes the basic instrumentation.

The various detectors are

- UV visible photometers
- Refractive index detector
- Flourimetric detector
- Conductivity detector
- Amperometric detector
- PDA

Detector electronic integrators and computing integrators are widely used today in HPLC for measuring peak areas. These devices automatically sense peaks and print out the areas in numerical forms. With the help of peak areas and height values, the peak width can be calculated (considering the peak as a triangle) and it can also be used for the calculation of number of theoretical plates.

Quantitation methods in HPLC

Peak heights or peak area measurements only provide a response in terms of detector signal.

This response must be related to the concentration or mass of the compound of interest. To accomplish this, some type of calibration must be performed.

The four primary techniques for quantization are

a. Normalized peak area method.
b. External standard method.
c. Internal standard method.
d. Method of standard addition.

a) Normalized peak area method

The area percent of any individual peak is referred to as normalized peak area. This technique is widely used to estimate the relative amounts of small impurities or degradation compounds in a purified material and in this method the response factor for each component is identified.

b) External Standard method

This method includes injection of both standard and unknown, and the unknown is determined graphically from a calibration plot or numerically using response factors.

A response factor (RF) can be determined for each standard as follows.

RF = Standard area (peak height) / Standard Concentration

The external standard approach for most samples in HPLC that do not require extensive sample preparation.

c) **Internal Standard method**

Internal standard is a different component from the analyte but one that is well resolved in the separation. The internal standard should be chosen to mimic the behavior of the sample component. One of the main reasons for using an internal standard is for samples requiring significant pre treatment or preparation.

d) **Method of standard addition**

The method of standard addition can be used to provide a calibration plot for quantitative analysis. It is more often used in trace analysis.

An important aspect of the method of standard addition is that the response prior spiking additional analytes should be high enough to provide a reasonable S/N ratio (<10), otherwise the result will have poor precision. (**Vogel's 2003**).

VALIDATION

Validation is a key process for effective quality assurance. "Validation is established documented evidence, which provides specific a high degree of assurance that a process of equipment will consistently produce a product or result meeting its predetermined specifications and quality attributes".

Definition

USFDA defines validation as **"**established documented evidence which provides a high degree of assurance that a specific process will consistently produce a product of predetermined specifications and quality attributes".

EUGMP defines validation as "action of proving in accordance with the principle of Good manufacturing practice (GMP), that any material activity or system actually lead to expected result".

AUSTRALIAN GMP defines validation as "the action of proving that any material, process, procedure, system, equipment or mechanism used in manufacture or control can and will be reliable and achieve the desire and intended result".

Importance of validation

1. As the quality of the product cannot be always assured by routine quality
   control because of testing of statistically insignificant number of sample.
2. The validation should provide adequacy and reliability of a system or product to meet the predetermined criteria or attributes, providing high degree of confidence that the same level of quality is consistently build into each of finished product from batch to batch.

3. Retrospective validation is useful for trend comparison or results complaints to cGMP to cGLP.
4. For taking appropriate action in case of non-compliance.

Objectives of validation

The primary objective of validation is to form a basis for written procedure for production and process control which are designed to assure that the drug products have the identity, quality and purity they purport or are represented to possess.

1. Assurance of quality.
2. Government regulation.

Types of validation

The following are frequently required to be validated on a pharmaceutical process

1. Equipment validation
2. Process validation
3. Cleaning validation
4. Analytical method validation
5. Facility validation including utilities

ANALYTICAL METHOD VALIDATION

Method validation is a process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Analytical testing of a pharmaceutical product is necessary to ensure the purity, stability, safety and efficacy. Analytical method validation is an integral part of the quality control system.

PARAMETERS USED FOR ASSAY VALIDATION

The validations of the assay procedure are carried out using following parameters.

SPECIFICITY:

Specificity is the ability to asses unequivocally analyte in the presence of impurities, degradants, matrix, etc which may be expected to be present. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures.

PRECISION:

Definition

The precision of an analytical procedure express the closeness of the agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. The precision of an analytical procedure is usually expressed as the variance, standard deviation or co-efficient of variation of a series measurement.

System precision

A system precision is evaluated by measuring the peak response for the six replicable injection of the same standard solution prepared as per the proposed method .The %RSD is calculated and it should not be more than 2%.

Method precision

A method precision is evaluated by measuring the peak response for six replicate injection of six different weigh of sample solution prepared as per proposed method. The %RSD is calculated and it should not be more than 2%.

Determination

The precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation.

ICH Requirements

The ICH documents recommended that repeatability should be assessed using a minimum number of nine determinations covering the specified range of the procedure (I, e., three concentrations and there replicates of each concentrations or using a minimum of six determinations at 100% of the test concentration).

ACCURACY

Definition

The accuracy of an analytical procedure express the closeness of the agreement between the values which is acceptable either as conventional true value or an accepted reference value and the value found.

Determination

In case of assay of drug in a formulated product ,accuracy may be determined by application of the analytical method to synthetic mixtures of the drug product components to which the known amount of analyte have been added within the range of the method . If it is not possible to obtain all product components ,it may be acceptable either to add known quantities of the analyte to the drug product or to compare results with those of a second ,well characterized method ,the accuracy of which has been stated or defined .Accuracy studies for drug substance and drug product are recommended to be performed at the 80,100 and 120% level of label claim as stated in the guidelines for submitting samples and analytical data for method validation .At each recommended level studied ,replicate samples are evaluated. The RSD of the replicates will provide the analysis variation or how precise the test method

is. The mean of the replicates, expressed as %label claim, indicates how accurate the test method is.

ICH Requirements

The ICH documents recommended that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e., three concentration and three replicates of each concentration).

LINEARITY

Definition

The linearity of an analytical procedure is its ability (with in a given range) to obtain the test results which are directly proportional to the concentration (amount) of analyte in the sample.

Determination

Linearity of an analytical procedure is established minimum of five concentrations. It is established initially by visual examination of plot of signals as a function of analyte concentration of content .If there appears to be a linear relationship ,test results are established by appropriate statistical methods(i.e., by calculation of the regression line by the method of least squares).

LIMIT OF DETECTION (LOD)

Definition

LOD is the lower concentration of the substance that the method can detect but not necessarily quantify.LOD simply indicates that the sample below or above a certain level.

Determination

For non instrumental methods, the detection limit is generally determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be reliably detected.

ICH Requirements

The ICH describes a common approach, which is to compare measured signal from samples with known concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be detected is established.

Typically acceptable signal-to-noise ratios are 2:1 or 3:1.

LIMIT OF QUANTITATION (LOQ)

Definition

LOQ is the lowest concentration of the substance that can be estimated quantitatively with acceptable precision, accuracy and reliability by the proposed method. LOQ is determined by the analysis of samples containing decreasing known quantity of the substance and determining the lowest level at which acceptable level of accuracy and precision is attained.

Determination

For non-instrumental methods, quantization limit is generally determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision.

RANGE

Definition

The range of an analytical procedure is the interval between the upper and the lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

Determination

The range of the method is validated by verifying that the analytical method provides acceptable precision, accuracy and linearity when applied to samples containing analyte at the extremes of the range as well as within the range.

ROBUSTNESS

Definition

The robustness of an analytical procedure is a measure of its capacity to remain unchanged by small but deliberately variations in method parameters and provides an indication of its reliability during normal usage.

Determination

The robustness of method determined by performing the assay by deliberately altering parameters(change in flow rate ±10%,change in mobile phase ratio ±2,change in pH of mobile phase ±0.2,change in wave length detection ±5nm, change in temperature ±1 to 5^0) that the results are not influence by the changes in the above parameters.

RUGGEDNESS

Definition

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the samples under a variety of conditions, such as different laborites, different analyst, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days etc.

Determination

The ruggedness of analytical method is determined by the analysis of aliquots from homogenous lots in different laboratories, by different analysis, using operational and environmental condition that may differ but are still within the specified parameters of the assay .The degree of reproducibility of the results is that determined as a function of assay variables. This reproducibility may be compared to the precision of assay under normal condition to obtain a measure of the ruggedness of the analytical method.

SAMPLE SOLUTION STABILITY

Solution stability of the drug substance or drug product after preparation according to the test method should be evaluated. Most laboratories utilize auto samples with overnight runs and the sample will be in solution for hours in the laboratory environment before the rest procedure is completed. This is concern especially for drugs that can undergo degradation by hydrolysis, photolysis, and adhesion to glassware.

SYSTEM SUITABILITY SPECIFICATION AND TESTS

The accuracy and precision of HPLC data collected begin with a well behaved chromatographic system. The system suitability specifications and tests are parameters that provide assistance in achieving this purpose.

It consists of following factors:

1. Capacity factor
2. Precision\Injection repeatability
3. Relative retention
4. Resolution
5. Tailing factor
6. Theoretical plate number

1. Capacity factor (K')

$$K' = (tR\text{-}tO/tf)$$

The capacity factor is a measure of where the peak of interest is located with respect to the void volume i.e., elution time of the non retained components.

2. Precision/Injection repeatability (RSD)

Injection precision expressed as RSD (relative standard deviation) indicates the performance of the HPLC which includes the pumping, column and the environmental conditions, at the time the samples are analyzed .It should be noted that sample preparation and manufacturing variations are not considered.

3. Relative retention (α)

$$\alpha = K'1/K'2$$

Relative retention is a measure of the relative location of two peaks. This is not an essential parameter as long as the resolution (Rs) is stated.

4. Resolution (Rs)

$$Rs = (t_{R2}\text{-}t_{R1})/(1/2)(t_{w1}+t_{w2})$$

Rs is a measure of how well two peaks are separated. For reliable quantitation well separated peaks are essential for quantitation. This is a very useful parameter if potential inference peaks (s) may be concern.

5. Tailing factor

$$T = Wx/2f$$

The accuracy of quantitation decreases with increases in peak tailing because of the difficulties encountered by the integrator in determine where/when the peak ends and hence the calculation of the area under the peak. Integrator variables are present by the analyst for optimum calculation of the area for the peak of interest. If the integrator is unable to determine exactly when an upslope for down slope occurs, accuracy drops.

6. Theoretical plate number (N)

$$N = 16(tR/tw)^2 = L/H$$

Theoretical plate number is a measure of column efficiency, that is, how many peaks can be located per unit run-time of the chromatograph.

N- Constant for each peak on the chromatogram with a fixed set of operating conditions.

H- Height equivalent of a theoretical plate.

L- Length of column.

LITERATURE REVIEW

K.S. LAKSHMI et al., (2009) have been developed a simple, sensitive and rapid reverse phase high performance liquid chromatographic method was developed for the estimation of Metformin Hcl (MET) and Pioglitazone (PIO) in pure and in pharmaceutical dosage forms. A Gemini C18 column (150x4.6mm, 5µ) was used with a mobile phase containing a mixture of Acetonitrile and Ammonium Acetate buffer (pH-3) in the ratio of 42: 58. The flow rate was 0.3ml/min and effluents were monitored at 255nm and eluted at 5.17min (MET) and 8.1min (PIO). Calibration curve was plotted with a range from 0.5-50 µg/ml for MET and 0.3-30 µg/ml for PIO. The assay was validated for the parameters like accuracy, precision, robustness and system suitability parameters. The proposed method can be useful in the routine analysis for the determination on metformin and pioglitazone in pharmaceutical dosage forms.

S Havele et al., (2010) have developed a simple, rapid, and precise reversed-phase high-performance liquid chromatographic method for simultaneous analysis of metformin hydrochloride, gliclazide, and pioglitazone hydrochloride in a tablet dosage form has been developed and validated. Chromatography was performed on a 25 cm × 4.6 mm i.d., 5-□m particle, C18 column with 85:15 (v/v) methanol: 20 mM potassium dihydrogen phosphate buffer as mobile phase at a flow rate of 1.2 ml/min. UV detection at 227 nm; metformin hydrochloride, gliclazide, and pioglitazone hydrochloride were eluted with retention times of 2.15, 3.787, and 4.57 min, respectively. The method was validated in accordance with ICH guidelines. Validation revealed the method is specific, rapid, accurate, precise, reliable, and reproducible. Calibration plots were linear over the concentration ranges 50– 250 □g/ml for metformin hydrochloride, 3.0 –15.0 □g/ml for gliclazide, and 2–10 □g/ml for pioglitazone hydrochloride. Limits of detection were 0.20, 0.04, and 0.10 □g/ml and limits of quantification were 0.75, 0.18, and 0.30 □g/ml for metformin hydrochloride, gliclazide, and pioglitazone hydrochloride, respectively. The high recovery and low coefficients of variation confirm the

suitability of the method for simultaneous analysis of the three drugs in tablets. Statistical analysis proves that the method is suitable for the analysis of metformin hydrochloride, gliclazide, and pioglitazone hydrochloride as a bulk drug and in pharmaceutical formulation without any interference from the excipients. It may be extended to study the degradation kinetics of three drugs and also for its estimation in plasma and other biological fluids

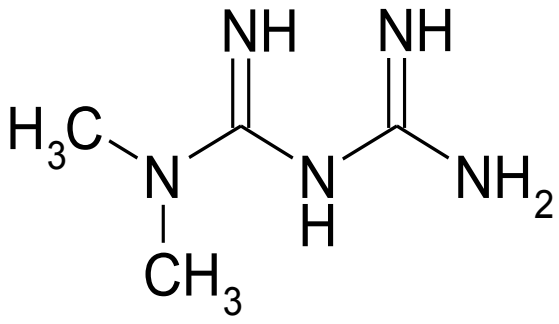
G Mubeen et al., (2010) have been developed a simple Spectrophotometric method has been developed and validated for the estimation of Metformin hydrochloride in bulk and in tablet formulation. The primary amino group of Metformin hydrochloride was oxidized using hydrogen peroxide to form a yellow chromogen , which is determined spectrophotometrically at 400 nm. It obeyed Beer's law in the range of 4-26mcg/ml.The percentage recovery of the drug for the proposed method ranged from 99-101.3% indicating no interference of the tablet excipients. The proposed method was found to be accurate and precise for routine estimation of Metformin hydrochloride in bulk and in tablet dosage forms.

MARIA-CRISTINA RANETTI et al., (2009) have been developed a simple HPLC method for the simultaneous determination of metformin (MTF) and gliclazide (GCZ) in the presence of glibenclamide, in human plasma, for the clinical monitoring of MTF and GCZ after oral administration or for bioequivalence studies. Ion-pair separation followed by UV detection performed on deproteinised plasma samples was chosen for the determination of metformin and gliclazide.The mobile phase was acetonitrile: methanol (1:1v/v) and sodium dodecylsulphate 5mM, pH=3.5 with H3PO4 85% and gradient elution. The eluent was monitored at 236 nm. The calibration curve was linear within the range of 0.05-5.00 μg/mL (r2=0.99, n=6). The lowest limit of quantification (LLOQ) was 50 ng/mL for metformin and 49 ng/mL for gliclazide.The proposed method was validated and proved to be adequate for metformin and gliclazide clinical monitoring, bioavailability and bioequivalence studies.

DRUG PROFILE

METFORMIN HCL

Structure



Molecular Formula : $C_{14}H_{11}N_5$.HCL

Molecular weight : 165.62g/mol

IUPAC Name : :N,N-dimethylimidodicarbonimidic diamide

Solubility **:** Freely soluble in water; slightly soluble in Ethanol;

Category : Anti -diabetic

Dose 25 to 100 mg

Mechanism of action:

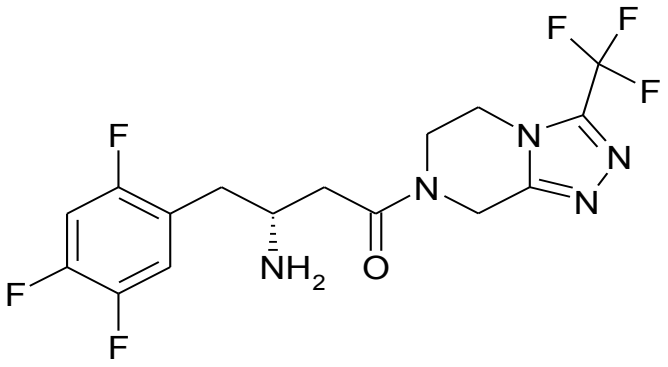
Metformin improves hyperglycemia primarily through its suppression of hepatic glucose production (hepatic gluconeogenesis. The "average" person with type 2 diabetes has three times the normal rate of gluconeogenesis; metformin treatment reduces this by over one third. Metformin activates AMP-activated protein kinase (AMPK), a liver enzyme that plays an important role in insulin signaling, whole body energy balance, and the metabolism of glucose and fats, activation of AMPK is required for metformin's inhibitory effect on the production of glucose by liver cells. Research published in 2008 further elucidated metformin's mechanism of action, showing activation of AMPK is required for an increase in the expression of SHP, which in turn inhibits the expression of the hepatic gluconeogenic genes PEPCK and Glc-6-Pase. Metformin is frequently used in research along with AICAR as an AMPK agonist. The mechanism by which biguanides increase the activity of AMPK remains uncertain; however, research suggests that metformin increases the amount of cytosolic AMP (as opposed to a change in total AMP or total AMP/ATP.

Adverse effects:

The most common adverse effect of metformin is gastrointestinal upset, including diarrhea, cramps, nausea, vomiting and increased flatulence; metformin is more commonly associated with gastrointestinal side effects than most other antidiabetic drugs.

SITAGLIPTIN

Structure:



Molecular formulae : $C_{16}H15F_6N_5O$

Molecular Weight : 407.314

IUPAC Name : (R)-4-oxo-4-[3-(trifluoromethyl)-5,6 dihydro[1,2,4]triazolo[4,3-a]pyrazin 7(8H)-yl]-1-(2,4,5-)butan-2-amine.

Category : Antidabetic

Dose : 25 to 100 mg.

Solubility : Soluble in water and N,N Dimethyl formamide;

Storage : Store in well-closed containers.

PHARMACOLOGY:

Mechanism of action:

Sitagliptin works to inhibit the enzyme dipeptidyl peptidase 4 (DPP-4). This enzyme breaks down the incretins GLP-land GIP, gastrointestinal hormones released in response to a measure. By preventing GLP-1 and GIP inactivation, they are able to potentiate the secretion of insulin and suppress the release of glucagon by the pancreas. This drives blood glucose levels towards normal. As the blood glucose level approaches normal, the amounts of insulin released and glucagon suppressed diminishes, thus tending to prevent an "overshoot" and subsequent low blood sugar (hypoglycemia) which is seen with some other oral hypoglycemic agents

Side effects:

The most common side effects of sitagliptin are abdominal pain, nausea diarrhea, vomiting and hypoglycemia. Lactic acidosis is a serious side effect of metformin that occurs in one out of every 30,000 patients and is fatal in 50% of cases. The symptoms of lactic acidosis are weakness, trouble breathing, abnormal heartbeats, unusual muscle pain stomach discomfort, light-headedness and feeling cold. Patients at risk for lactic acidosis include those with reduced function of the kidneys or liver, congestive heart failure severe acute illnesses, and dehydration.

AIM AND PLAN OF WORK

The drug analysis is playing an important role in the development of drugs, their manufacture and therapeutic use. For the simultaneous estimation of drugs present in dosage forms , lot of suitable methods are adopted like uv – spectrophotometer , HPLC , HPTLC etc .These methods are powerful and rugged method .They are also extremely precise, specific, accurate, linear and rapid.

A pharmaceutical industry depends upon quantitative chemical analysis to ensure that the raw material used and the final product obtained meets the required specification. The drugs will occur as a single component or multi component dosage forms. The later proves to be effective due to its combined mode of action on the body.

The number of drugs or drug formulations introduced into the market is increasing at a fast rate .These may be either new entries in the market or structural modification of the existing drugs or novel dosage forms or multi component dosage forms .The complexity in the dosage forms, including that of the multi component dosage forms creates considerable challenges to the analytical chemist during the development of assay procedure for its accurate estimation. The estimation of individual drugs in these multi component dosage forms becomes difficult due to tedious extraction or isolation procedure.

The combination of Metformin HCL and Sitagliptin was selected for the present study.

According to the literature survey conducted, it was observed that no method was reported in RP-HPLC for the estimation of individual drug carried out. Hence present study aims to develop an accurate, precise, specific, linear, simple, rapid, validated and cost effective analytical method for Metformin HCL and Sitagliptin in tablet dosage form by RP-HPLC method. The scope of our work extends to validate for the developed method as per ICH guidelines.

RP-HPLC method development was obtained as

- ✓ Selection and optimization of mobile phase and stationary phase.
- ✓ Selection of detector wavelength.
- ✓ Selection of extraction procedure.
- ✓ Optimization of chromatographic condition.
- ✓ Estimation of Metformin HCL and Sitagliptin
- ✓ Method validation.

MATERIALS AND METHODS

Instrumentation:

S.No.	Name of instrument	Model	Make
1	Semi micro balance	CPA225D	Sartorius
2	pH meter	Metler Toledo	Thermo Orion
3	HPLC	LC-20 AT	Shimadzu
4	C 18 Column	Phenomenex	Gemini
5	Sonicator	USB	Spectro lab
6	UV	1700 series	Shimadzu

Chemicals and reagents:

S.No.	Chemicals/Reagents	Make/grade
1	Glacial acetic acid	Merck(HPLC Grade)
2	Dipotassium hydrogen phosphate	Merck(GR Grade)
3	Methanol	Merck(GR Grade)
4	water	Merck(GR Grade)

Working/Reference Standards:

S.No	Name of Working/reference standards	% Purity
1	Metformin HCL Working standard	99.65
2	Sitagliptin Working standard	99.61

Filters:

S .No	Name of the filter
1.	0.45 m GHP membrane filter(Manufactured by PALL)

METHOD DEVELOPMENT GUIDE

Information on sample, define separation goals.

↓

Need for special procedure, sample treatment.

↓

Choose detector and detector settings.

↓

Choose the method: preliminary run: estimate the best separation condition

↓

Optimize separation conditions.

↓

Validate the method.

Table-1

Goal	**Comment**
Resolution	Precise and rugged quantitative analysis requires that R_s be greater than 1.5.
Separation time	<5-10 min is desirable for routine procedures
Quantitation	≤2% for assays ;≤5% for less demanding analysis ≤15% for trace analyses.
pressure	<150 bar is desirable, <200 bar is usually essential (new column assumed).
Peak height	Narrow peaks are desirable for large signal /noise ratios.
Solvent consumption	Minimum mobile-phase use per run is desirable.

POLARITY OF COMMON ORGANIC FUNCTIONAL GROUPS AND SOLVENTS

Functional Groups	Non-Polar	Solvent
Aliphatic hydrocarbons		Hexane
Olefines		Carbon tetrachloride
Aromatic hydrocarbons		Ester
Halides		Benzene
Sulphides		Methyl chloride
Ethers		THF
Nitro components		Isopropanol
Esters, aldehydes, ketones		Chloroform
Alcohols, amines		Ethylacetate
Sulphones		Acetonitrile
Sulphoxides		Methanol
Amides		Water
Carboxylic acids		
	Polar	

OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

1. Selection of wavelength for detection of components

Solution of Metformin HCL and Sitagliptin were scanned in the UV region and spectrum was recorded .The solvent used was 0.02M dipotassium hydrogen phosphate,and acetonitrile in the ratio 55:45. It was seen that at 260nm all compounds have good absorbance, which can be used for the estimation of compounds by HPLC.

2. Selection of chromatographic method

Proper selection of the method depends on the nature of the sample (ionic or ionisable or neutral molecules), its molecular weight, pka value and stability .The drugs selected in the present study are polar and so reversed phase or ion exchange chromatography can be used. The reverse phase HPLC was selected for the initial separation because of its simplicity and suitability.

For the literature survey and with knowledge of properties of the selected drugs, Phenominex Gemini C18 (250 × 4.6mm) 5µ column was chosen as stationary phase and mobile phase with different compositions such as Acetonitrile was used. The separations were not observed so use of buffer was finalized.

For all the data observed, obtained and available the initial separation condition were set to work around.

3. Initial separation condition

The following chromatographic conditions were fixed initially to improve the separation of both drugs.

Instrument : Shimadzu prominence

Column : Phenomenex Gemini C18 (250 × 4.6mm), 5µ.

Column oven temperature : Ambient

Wavelength : 260nm

Flow rate : 1.2ml/min

Injection volume : 20µl

Run time : 10 min

Mobile phase : Solvent A - Buffer

Solvent B - Methanol

Solvent C - Acetonitrile

Solvent Ratio : 30:35:35% $^{V}/_{V}$ of A: B: C

TRAILS

Trail-1

The trail was performed using Mobile phase in the ratio 30:35:305using Phenomenex C18 (250 x 4.6 mm, 5µ) with flow rate of 1.2ml/min.

In this trail, the retention time ofMetformin HCL and Sitagliptin peak was found to be 0.9, and 4.0 min respectively.

Trail-2

The trail 2 was performed using Mobile phase in the ratio 30:40:30 using Phenomenex C18 (250x 4.6 mm, 5µ) with flow rate of 1.2 ml/min.

In this trail, the retention time of Metformin HCL and Sitagliptin peak was found to be 0.7 and2.4 min. respectively.

Trail-3

The trail 3 was performed using Mobile phase in the ratio 55:45of using Phenomenex C18 (250 x 4.6 mm, 5 µ) with flow rate of 1 ml/min.

In this trail, the retention time of Metformin HCL and Sitagliptin peak was found to be 4.28and 7.485min respectively.

Trial-4:

The trail 4 was performed using Mobile phase in the ratio of 40:40 :20using Phenomenex (250 x 4.6 mm, 5 µ) with flow rate of 1 ml/min.

In this trail, only two peaks were shown at 2 and 3.2 min.

Out of 4 trails made in the lab, the 3th trail was selected for further studies because when compared to other trails, the 3th trail was found to be having less retention time and within the acceptance criteria.

4. Effect of ratio of mobile phase

Under the chromatographic conditions mentioned above the different ratios of mobile phase were tried .The chromatograms where observed for each of the trials, out of which 30: 35:35 i.e.; 30 Buffer: 35 Methanol: 35 Acetonitrile was selected as the separation was achieved in minimum retention time.

5. Effect of pH of mobile phase

Several trials were made using different buffer solutions of pH range .The best separation was achieved when adjusted the pH to 4.5 with orthophosphoric acid.

6. **Effect of flow rate on separation**

The mobile phase consisting of buffer: methanol: acetonitrile was used and the chromatograms were recorded at flow rates of 1ml/min, 1.2ml/min. The sharpest peaks were obtained with 1.5ml/min flow rate.

7. Effect of column (stationary phase) on separation

At the chromatographic conditions of mixed solutions, combination of Metformin HCL and Sitagliptin were injected and chromatograms were obtained using C-18 columns.

8. Reference standards

Keeping all other above fixed conditions, external standard was used.

9. Optimized condition

The following optimized parameters were used in a final method for the simultaneous estimation of Metformin HCL and Sitagliptin.

Instrument	: Shimadzu Prominence
Column	: Phenominex C18 (250 × 4.6mm), 5µ.
Column oven temperature	: Ambient
Wavelength	: 260nm
Flow rate	: 1ml/min
Injection volume	: 20µl
Run time	: 10 min
Mobile phase	: Solvent A - Buffer
	Solvent B- Acetonitrile
Solvent Ratio	: 55:45% $^{V}/_{V}$ of A: B

QUANTITATION

Samples obtained from local market.

Metformin HCL -500mg

Sitagliptin-50mg

Preparation of Dipotassium hydrogen phosphate buffer pH 4.5:

Prepare about 0.02M dipotassium hydrogen phosphate in a suitable conical flask and adjust the pH to 4.5 with orthophosphoric acid.(0.02M of di potassiumhydrogen phosphate is prepared by taking 1.3602mg of dipotassiumhydrogen phosphate in a volumetric flask , and make up to 1L with water).

Preparation of mobile phase:

Prepare a mixture of buffer 4.5 pH, and acetonitrile in the ratio 55:45 filter through 0.45µ membrane filter and degas it.

Diluent preparation: Buffer 4.5 pH, and acetonitrile in the ratio 55:45

Standard preparation:

Weigh accurately about 50mg of Metformin, 50mg Sitagliptin working standard to a 100ml volumetric flask.Dissolve it completely and sonicate it. Make up to 100ml mobile phase. Take 3ml from the above flask and make up to 50ml with mobile phase.

Sample preparation:

Weigh accurately 20 tablets equivalent to 92.4mg to a 100ml volumetric flask.mobile phase to dissolve it completely and sonicate for 10min with intermediate shaking Make up to 100ml with mobile phase and filter through 0.45µ GHP filter. Further dilute 3ml with 50ml mobile phase.

Calculation:

Determine the % amount of Metformin HCL and Sitagliptin in tablets according to the following formula.

$$\%\text{Assay} = \frac{AT \times W\,R \times 3 \times 100 \times 50 \times PR \times \text{Average Weight}}{AR \times 100 \times 50 \times WT \times 3 \times 100 \times LA} \times 100$$

Where,AT = Area in the test solution

AR = Area in the standard solution

WR = Weight of standard solution (mg)

WT = Weight of sample in test preparation (mg)

PR = Purity of working standard (%)

LA = Labeled amount of Metformin HCL and Sitagliptin per Tablets.

VALIDATION OF THE DEVOLOPED METHOD

SPECIFICITY

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix. The other component may include excipients, impurities, degradation product etc.

Peak purity test may be useful to show that the analyte chromatographic peak is not contributed by more than one component (e.g. .diode array, mass, spectroscopy).

Standard preparation:

Weigh accurately about 50mg of Metformin, 50mg Sitagliptin working standard to a 100ml volumetric flask.Dissolve it completely and sonicate it. Make up to 100ml mobile phase. Take 3ml from the above flask and make up to 50ml with mobile phase.

Table - 2

Sample	**Metformin HCL**		**Sitagliptin**	
	Avg area	**%Drug Content**	**Avg area**	**%Drug Content**
Standard	352.914	100.18	440.46	99.67
Sample	354.469		436.16	

Acceptance Criteria:

There is no interference in the standard peak.

LINEARITY:

Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range.

Linearity is generally reported as the variance of the slope of the regression line. Linearity should be evaluated by visual inspection of a plot of signal as a function of analyte concentration. The correlation coefficient, y-intercept, slope of the regression line and the residual sum of squares should be calculated.

Linearity of Metformin HCL and Sitagliptin

Weigh accurately about 50mg of Metformin, 50mg Sitagliptin working standard to a 100ml volumetric flask.Dissolve it completely and sonicate it. Make up to 100ml mobile phase. Take 3ml from the above flask and make up to 50ml with mobile phase.

Table - 3

LEVEL	**Metformin**	**Sitagliptin**
80%	1773.542	164.743
90%	1996.980	1848.657
100%	2221.836	2053.140
110%	2466.998	2251.260
120%	2663.495	2478.061
Y – intercept	1548	1430
Slope	22.48	207.7
Correlation Coefficient	0.999	0.999

Linearity Graph

RANGE:

Range is the interval between the upper and the lower levels of analyte that have been demonstrated to be determined with precision, accuracy and linearity using the method.

The range is normally expressed in the same unit as the test results obtained by the method. The ICH guideline specify a minimum of five

concentration levels, along with certain minimum specified ranges .For assay tests the minimum specified range is 80 – 120%of the target concentration .

Preparation of working standard solution

To get a concentration of 80%, 100%, 120%, of drug, pipette out 4ml, 5ml, 6ml, of mixed standard stock solution into separate 100ml volumetric flask and volume is made up with mobile phase. Further dilute 3ml of the solution to 100ml of mobile phase.

Acceptance Criteria:

- The %RSD for the individual recoveries of each level and mean recovery should not be more than 2.0%.
- The % recovery at each level and mean recovery should be in between 98.0% to 102.0%.

LIMIT OF DETECTION (LOD)

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, though not necessarily quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value.

ICH has recommended some method for determining the limit of detection. The method may be either instrumental or non-instrumental. They are

- Visual Evaluation
- Signal – to – Noise ratio convention
- Based on Standard deviation of the response and the slope of calibration curve

Limit of detection (LOD) based on standard deviation of the response and the slope of calibration curve.

$$LOD = \frac{3.3\ s}{S}$$

Where

s = Standard deviation of the response

S = Slope of calibration curve

Table - 4

Limit of detection study:

LOD	**Metformin HCL:(µg)**	**Sitagliptin(µg)**
1.	1.05	7.12

LIMIT OF QUANTITATION (LOQ)

The limit of Quantitation (LOQ) is defined as the lowest concentration of the analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method.

Limit of Quantitation (LOQ) is also based on standard deviation of the response and the slope of calibration curve.

$$LOQ = \frac{10\ s}{S}$$

Where,

s = Standard deviation of the response

S = Slope of calibration curve

Table - 5

Limit of Quantitation study:

LOQ	Metformin HCL(µg)	Sitagliptin(µg)
1.	5.6	3.5

PRECISION

Precision is the measure of the degree of repeatability of an analyte method under normal operation and is normally expressed as percent relative standard deviation for a significant number of the samples.

According to the ICH precision should be performed at three different levels: Repeatability, Intermediate precision, Reproducibility

System precision

The system precision was evaluated by measuring the peak response of Metformin HCL and Sitagliptin Hydrochlorothiazide, WS solution prepared as per the proposed method and chromatograms were recorded.

Determination

Weigh accurately about 50mg of Metformin, 50mg Sitagliptin working standard to a 100ml volumetric flask.Dissolve it completely and sonicate it. Make up to 100ml mobile phase. Take 3ml from the above flask and make up to 50ml with mobile phase.

s.no	Metformin	Sitagliptin
1	2051.31	2218.08
2	2061.14	2228.22
3	2047.51	2230.72
4	2055.83	2229.53
5	2043.94	2212.8
6.	2039.57	2217.84
AVG	2049.88	2222.86
STD/%RSD	0.33/0.33	0.37/0.38

Method Precision

Weigh accurately 20 tablets equivalent to 92.4mg to a 100ml volumetric flask.mobile phase to dissolve it completely and sonicate for 10min with intermediate shaking Make up to 100ml with mobile phase and filter through 0.45µ GHP filter. Further dilute 3ml with 50ml mobile phase.

Table - 6

Method Precision for Metformin HCL:

Sample. No	**% Assay**
Sample Preparation – 1	100.14
Sample Preparation – 2	100.1.8
Sample Preparation – 3	100.71
Sample Preparation – 4	100.76

Sample Preparation – 5	100.51
Sample Preparation – 6	100.56
Avg	100.76
SD	0.688
% RSD	0.70

Table - 7

Method Precision for Sitagliptin:

Sample. No	**% Assay**
Sample Preparation – 1	97.67
Sample Preparation – 2	98.60
Sample Preparation – 3	97.62
Sample Preparation – 4	98.65
Sample Preparation – 5	97.20
Sample Preparation – 6	97.24
Avg	97.80
SD	0.712
% RSD	0.730

Acceptance Criteria:

- The % RSD for the individual recoveries of each level and mean recovery should not be more than 2 %.
- The % recovery at each level and mean recovery should be in between 98.0% to 102%.

ACCURACY

Accuracy is the measure of exactness of an analytical method, or the closeness of agreement between the measured value and the value that is accepted either as a conventional, true value or an accepted reference value.

The accuracy may be determined by application of analytical method to an analyte of known purity (example: reference standard) and also by comparing the results of the method those obtained using an alternative procedure that has been already validated.

To document accuracy, the ICH guideline on methodology recommends collecting data from a minimum of nine determinations over a minimum of three concentration levels covering the specified range.

Result: Refer range of calculations.

Preparation of working standard solution

To get a concentration of 80%, 100%, 120% of drug, pipette out 4ml, 5ml, 6ml, of mixed standard stock solution into separate 100ml volumetric flask and volume is made with mobile phase. Further dilute 3ml this solution to 50ml with mobile phase.

Recovery values of Sitagliptin

Table 8

Concentration	**Avg Area**	**Amount Recovery**	**% Recovery**
80	1679.874	3.96	99.78
100	2086.258	4.96	99.24
120	2565.329	6.08	101.18
		Mean	100.06
		SD	0.79
		%RSD	0.79

Table – 9

Recovery values of Metformin

Concentration	**Avg Area**	**Amount Recovery**	**% Recovery**
80	1777.467	39.89	99.93
100	2210.760	49.50	99.05
120	2694.174	59.99	100.03
		Mean	99.66
		SD	0.44
		%RSD	0.44

Acceptance Criteria:

- The % RSD for the individual recoveries of each level and mean recovery should not be more than 2 %.
- The % recovery at each level and mean recovery should be in between 98.0% to 102%.

ROBUSTNESS

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters.

The robustness of a method is evaluated by varying method parameters such as percent organic solvent, pH, ionic strength or temperature and determining the effect on the results of the method. Robustness tests were generally introduced to avoid problems in linear laboratory studies and to identify the potentially responsible factors.

Determination of robustness

Robustness was performed by varying the

- P^H
- Flow rate

Table - 10

Robustness study for Metformin HCL and Sitagliptin**:**

Robustness Criteria	**RT of Metformin**	**RT of Sitagliptin**
Change in flow +0.2	3.707	6.100
Change in flow -0.2	4.790	7.560

Change in wavelength by -P^H	4.27	7.44
Change in wavelength by + P^H	4.28	7.48

Acceptance Criteria:

- Shall comply the system suitability parameters.
- Measured variation to be reported with appropriate recommendations

RUGGEDNESS

Ruggedness of analytical method is the degree of reproducibility of the results obtained by the analysis of the same samples under a variety of test conditions such as different laboratories, analysts, instruments, temperature, different days etc.

Determination of Ruggedness

The Ruggedness of an analytical method was determined by the analysis of aliquots from homogenous lots in different laboratories by different analysts using operational and environmental condition that may differ but are still within the specified parameters of the assay. The degree of reproducibility of the results is then determined as a function of assay variables. This reproducibility may be compared to the precision of assay under normal condition to obtain a measure of the ruggedness of the analytical method.

To determine the degree of reproducibility of the results by this method involved the studies of the analyst to analyst and day to day; that is to carry out precision study in six replicate of an assay of a single batch sample by two different analysts on two different days.

Ruggedness Interday Analysis Study :(Table –11)

Sample. No	% Assay of Metformin HCL	% Assay of Sitagliptin
Analyst – 1	98.9	102.0
Analyst – 2	99.2	100.1
Analyst – 3	99.7	101.4
Analyst – 4	99.6	101.3

Acceptance Criteria:

- The % recovery at each level and mean recovery should be in between 98.0% to 102%.

SYSTEM SUITABILITY

To verify whether the analytical system is working properly or it can give accurate and precise results, the system suitability parameters are to be set.

Inject separately 20 µL each of the following solutions into the HPLC.

Standard preparation:

Weigh accurately about 50mg of Metformin, 50mg Sitagliptin working standard to a 100ml volumetric flask.Dissolve it completely and sonicate it. Make up to 100ml mobile phase. Take 3ml from the above flask and make up to 50ml with mobile phase.

Table - 12

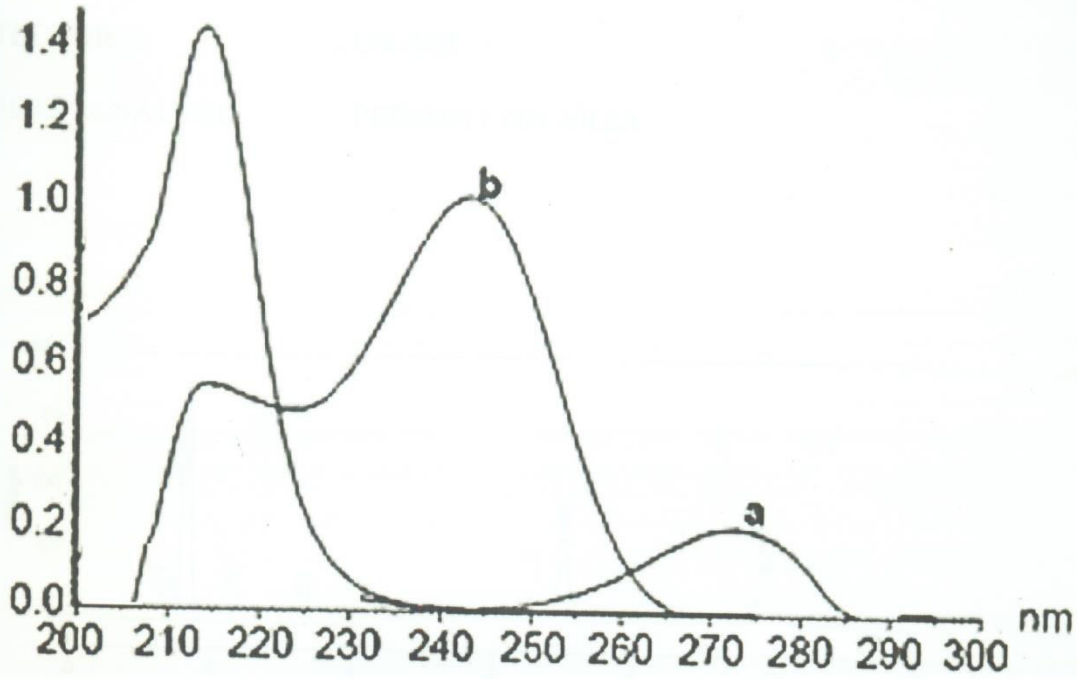
System suitability parameters	**Metformin HCL**	**Sitagliptin**
Tailing factor	1.056	1.000
No. of theoretical plates	9226	11340
Resolution	-	13.861

Acceptance criteria as follows:

In the chromatogram obtained with Standard,

- The % RSD of area of Metformin HCL and Sitagliptin in replicate injections of standard solution should not be more than 2.0.
- The tailing factor of Metformin HCL and Sitagliptinpeak should not be more than 2.0
- The theoretical plates of Metformin HCL and Sitagliptin peak should be more than 2000.

OVERLAID SPECTRA OF METFORMIN HCl & SITAGLIPTIN



CHROMATOGRAM-08

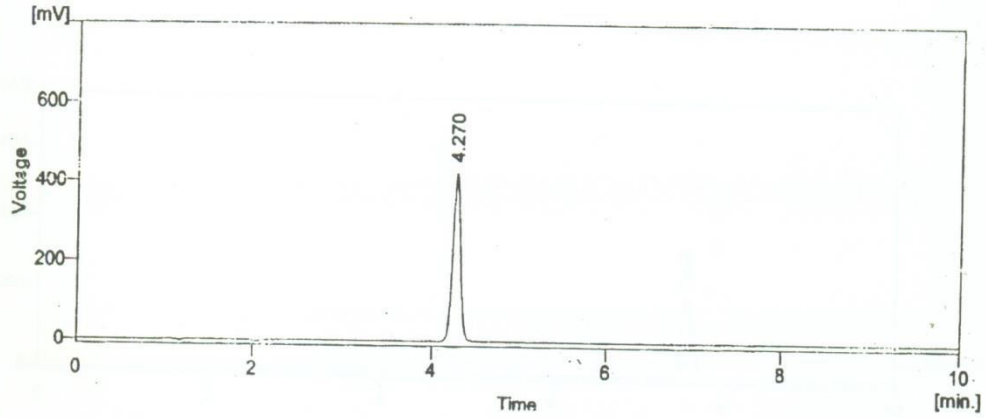
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : METFFORMIN HCL

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.270	2231.765	100.0
	Total	2231.765	100.0

CHROMATOGRAM-09

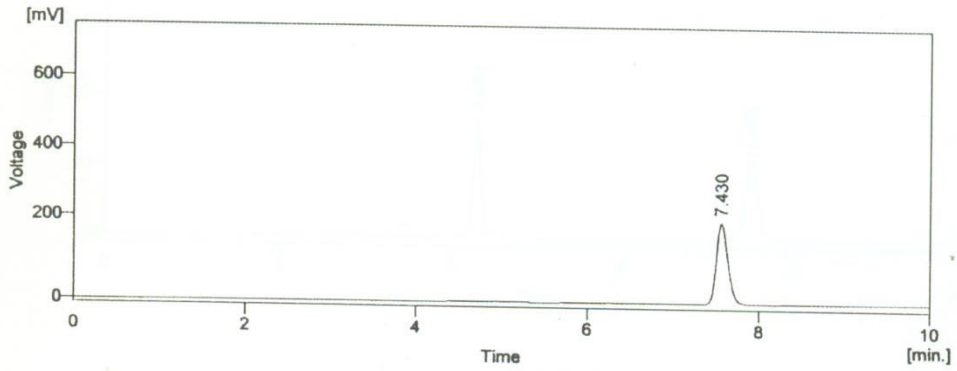
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : SITAGLIPTIN

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	7.430	2102.11	100.0
	Total	2102.11	100.0

CHROMATOGRAM-10

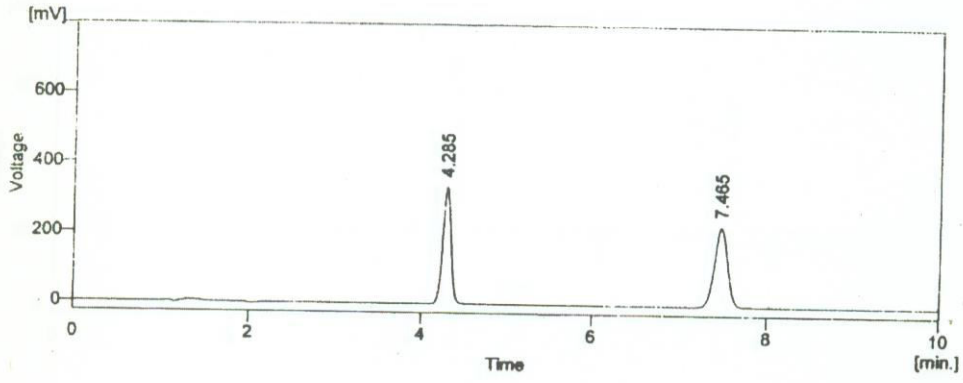
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : SYSTEM PRECISION-1

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.285	2218.085	52.0
2	7.465	2051.31	48.0
	Total	4269.395	100.0

CHROMATOGRAM-11

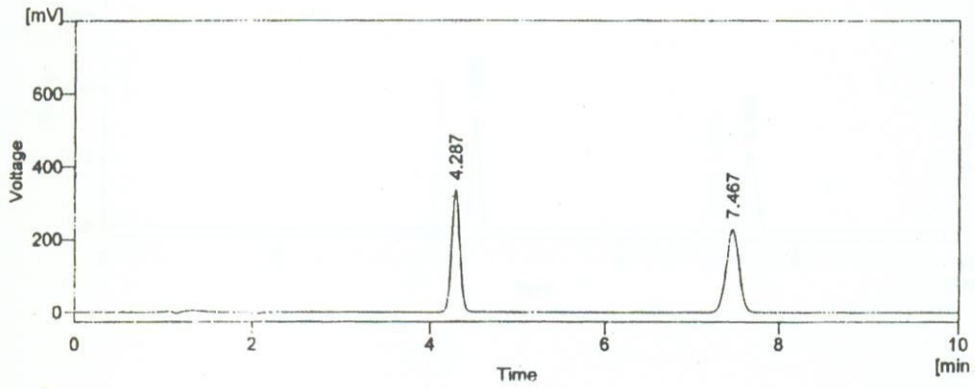
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : SYSTEM PRECISION- 2

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.287	2228.224	51.9
2	7.467	2061.147	48.1
	Total	4289.371	100.0

CHROMATOGRAM-12

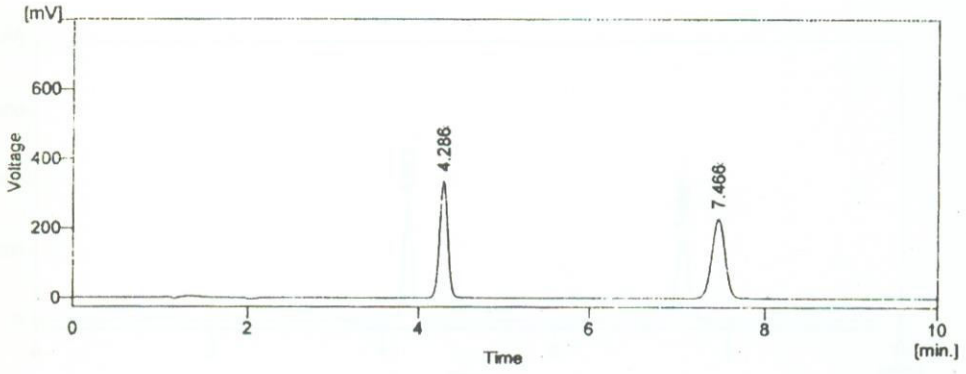
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : SYSTEM PRECISION- 3

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.286	2230.726	52.2
2	7.466	2047.512	47.8
	Total	4278.238	100.0

CHROMATOGRAM-13

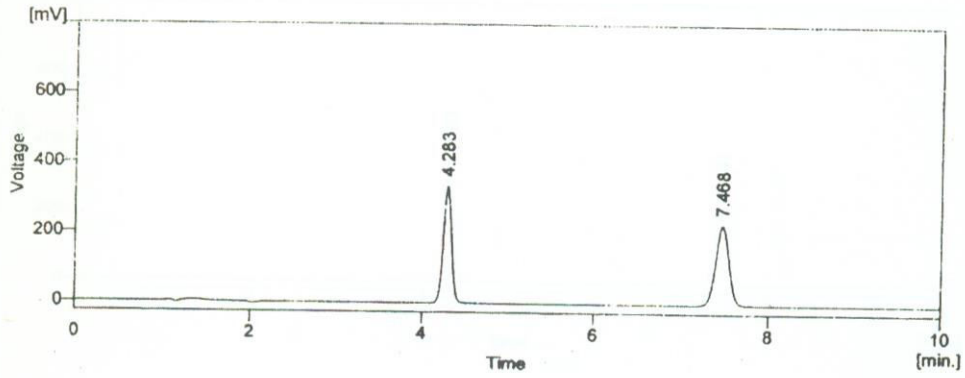
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : SYSTEM PRECISION-4

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.283	2229.530	52.1
2	7.468	2055.830	47.9
	Total	4285.360	100.0

CHROMATOGRAM-14

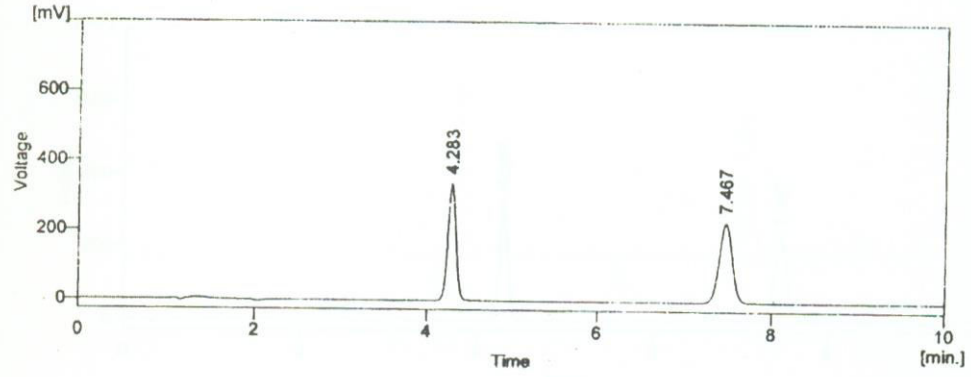
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : SYSTEM PRECISION- 5

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.283	2212.81	51.9
2	7.467	2043.94	48.1
	Total	4256.75	100.0

CHROMATOGRAM-16

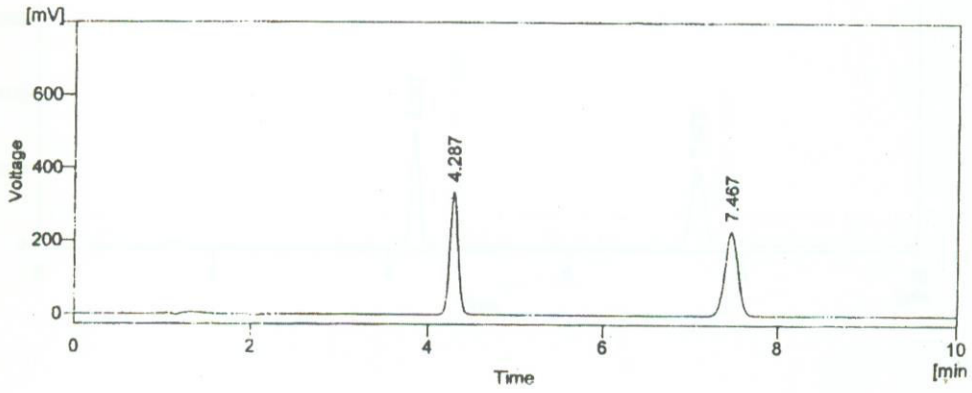
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : SYSTEM PRECISION- 6

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.287	2217.84	52.1
2	7.467	2039.57	47.9
	Total	4257.410	100.0

CHROMATOGRAM-17

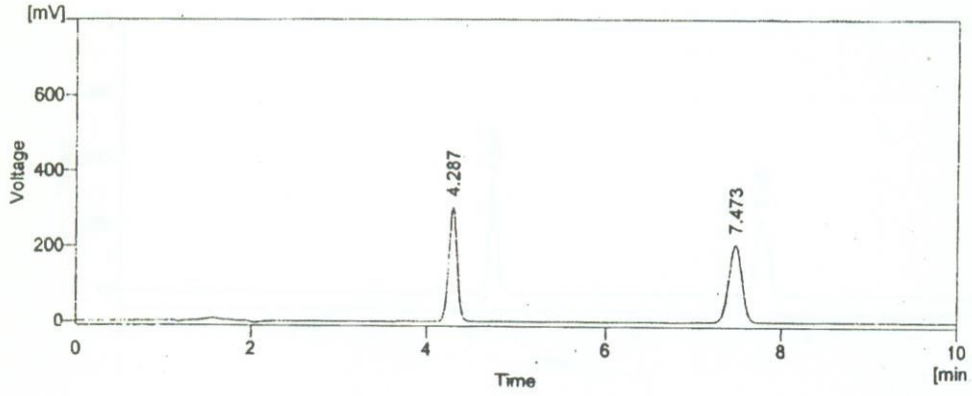
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : LINEARITY 1

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.287	1773.545	52.0
2	7.473	1640.741	48.0
	Total	3414.286	100.0

CHROMATOGRAM-19

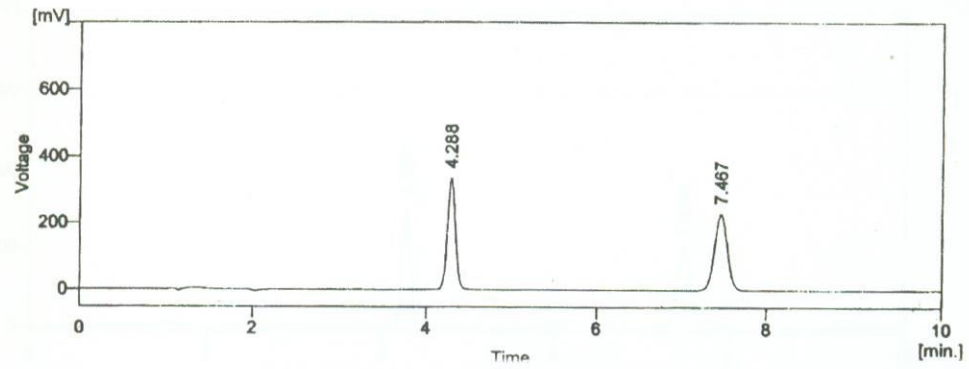
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : LINEARITY 3

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.288	2221.835	52.0
2	7.467	2053.143	48.0
	Total	4274.978	100.0

CHROMATOGRAM-18

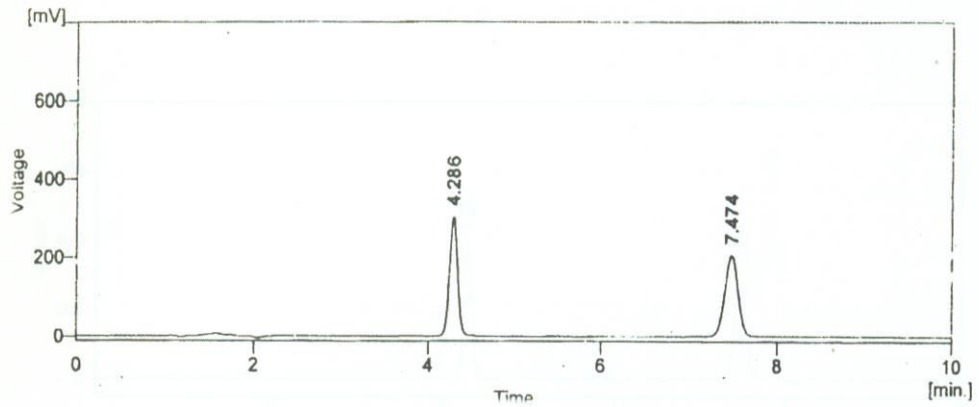
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : LINEARITY 2

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.286	1996.980	51.9
2	7.474	1848.658	48.1
	Total	3845.630	100.0

CHROMATOGRAM-20

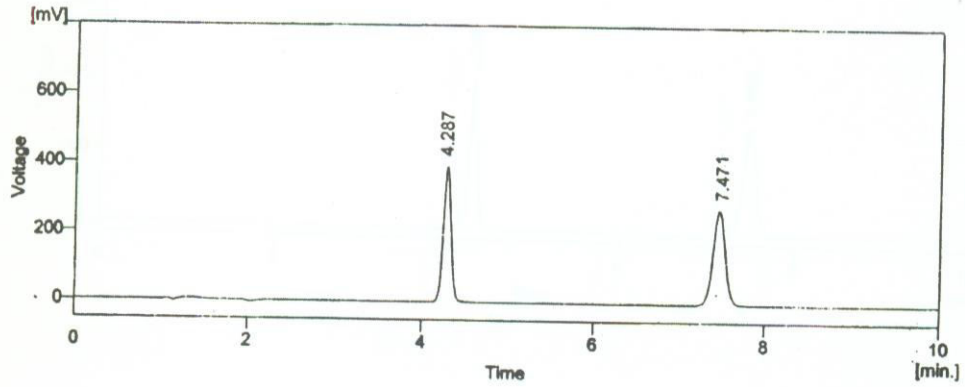
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : LINEARITY 4

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.287	2466.999	51.5
2	7.471	2251.240	48.5
	Total	4638.950	100.0

CHROMATOGRAM-21

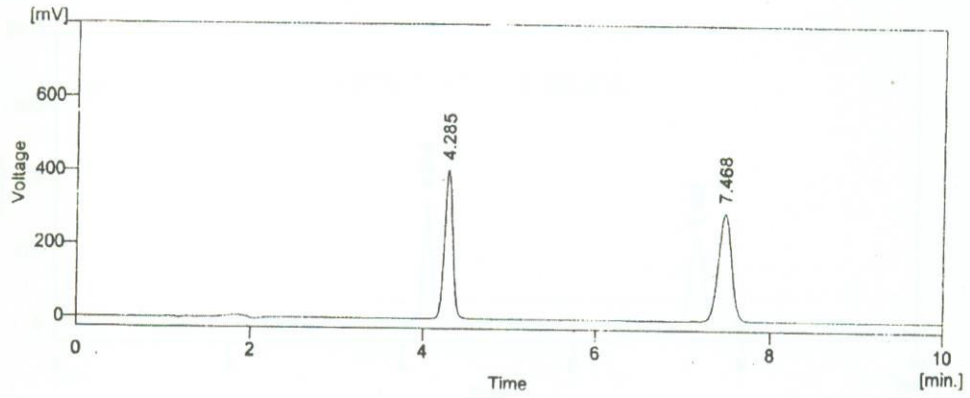
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : LINEARITY 5

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.285	2663.496	51.9
2	7.468	2478.061	48.1
	Total	5141.557	100.0

CHROMATOGRAM-21

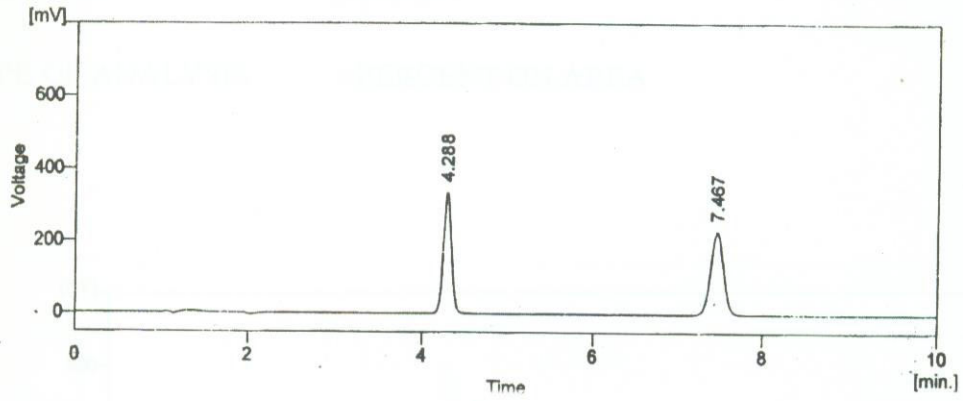
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : METHOD PRECISION-1

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.288	2235.846	52.2
2	7.467	2059..321	47.8
	Total	4287.167	100.0

CHROMATOGRAM-22

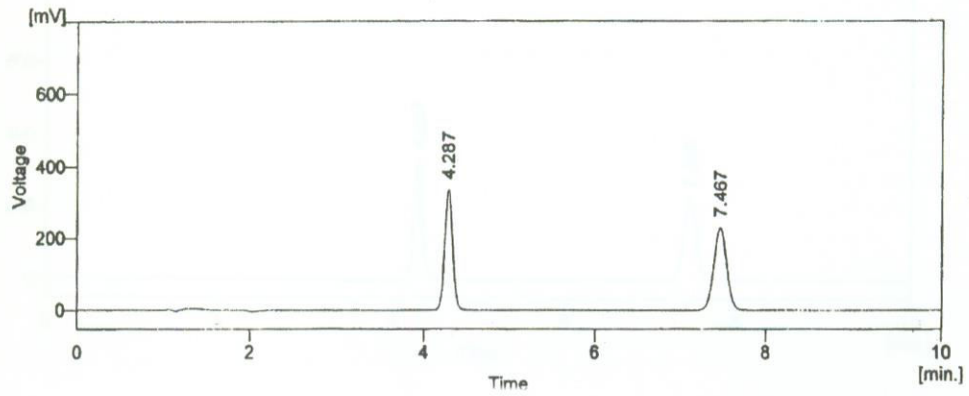
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : METHOD PRECISION-2

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.287	2247.379	52.3
2	7.467	2052.455	47.7
	Total	4299.834	100.0

CHROMATOGRAM-23

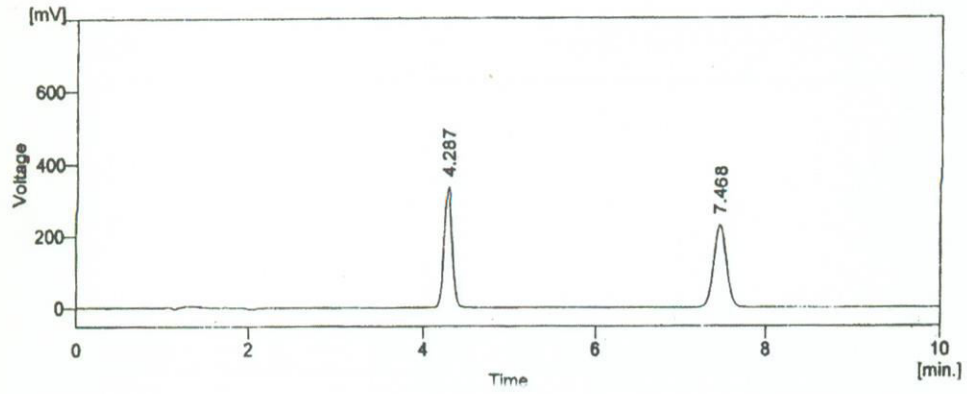
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : METHOD PRECISION-3

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.287	2265.832	52.6
2	7.468	2043.340	47.4
	Total	4309.172	100.0

CHROMATOGRAM-24

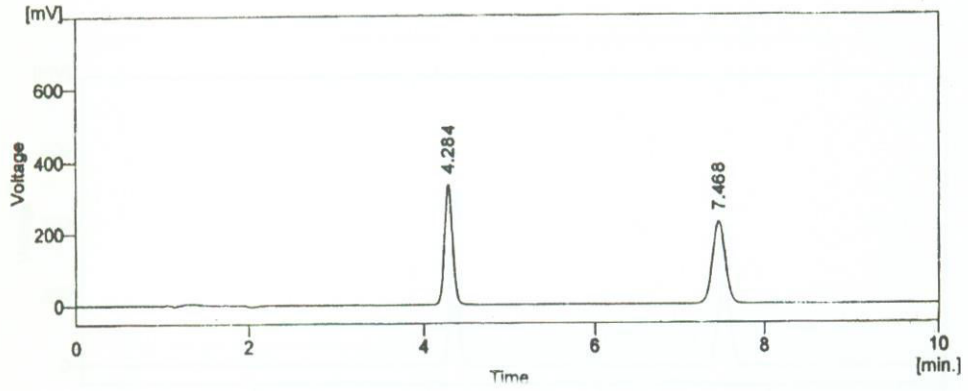
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : METHOD PRECISION-4

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.284	2255.483	52.5
2	7.468	2047.729	47.5
	Total	4303.212	100.0

CHROMATOGRAM-25

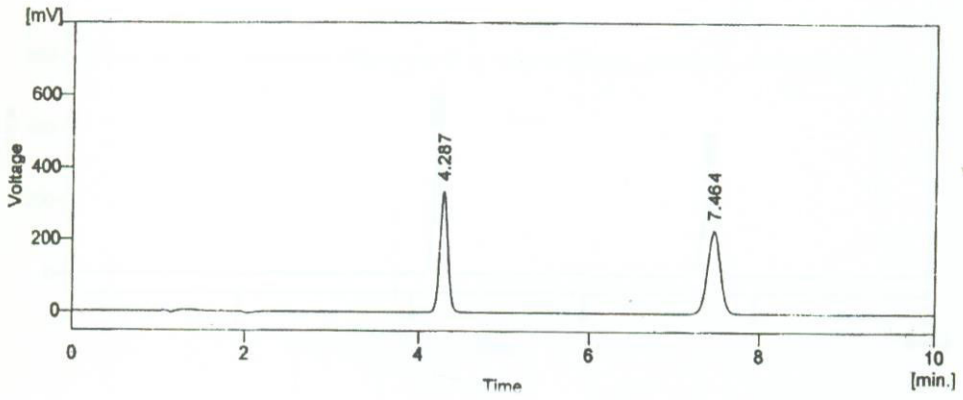
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : METHOD PRECISION-5

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time Min)	Area (mV)	Area (%)
1	4.287	2249.342	52.4
2	7.464	2049.374	47.6
	Total	4298.716	100.0

CHROMATOGRAM-26

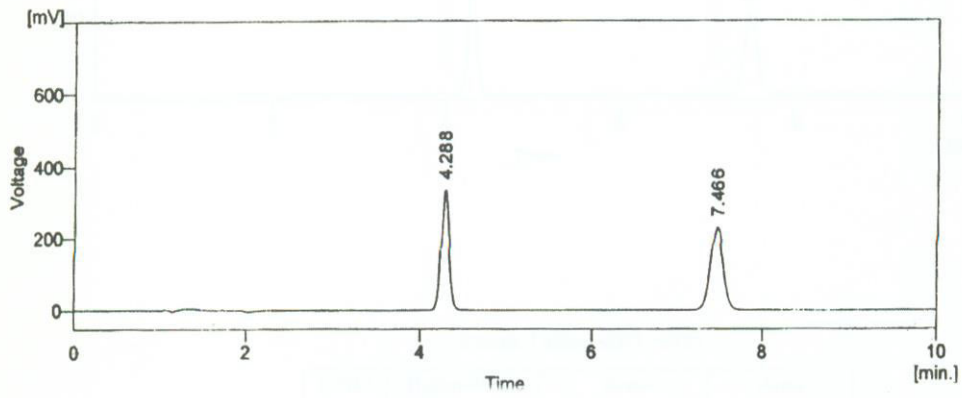
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : METHOD PRECISION-6

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.288	2242.736	52.3
2	7.466	2050.742	47.7
	Total	4293.478	100.0

CHROMATOGRAM-27

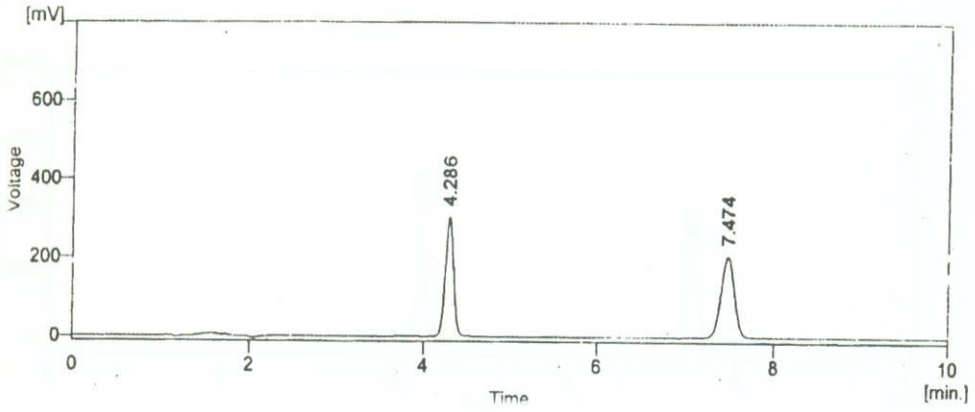
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : ACCURACY -80%

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.286	1996.980	51.9
2	7.474	1848.658	48.1
	Total	3845.630	100.0

CHROMATOGRAM-28

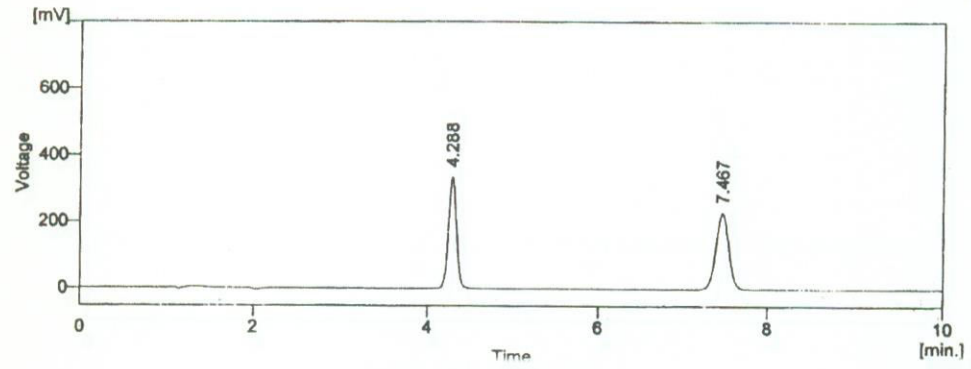
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : ACCURACY-100

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.288	2221.835	52.0
2	7.467	2053.143	48.0
	Total	4274.978	100.0

CHROMATOGRAM-28

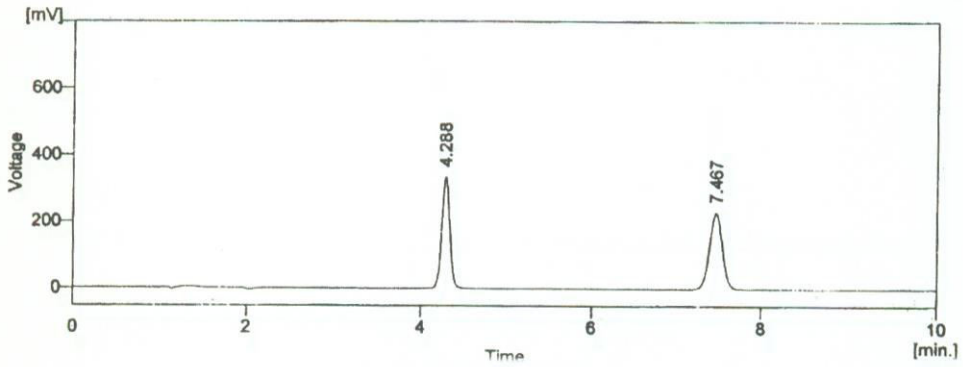
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : ACCURACY-100

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.288	2221.835	52.0
2	7.467	2053.143	48.0
	Total	4274.978	100.0

CHROMATOGRAM-29

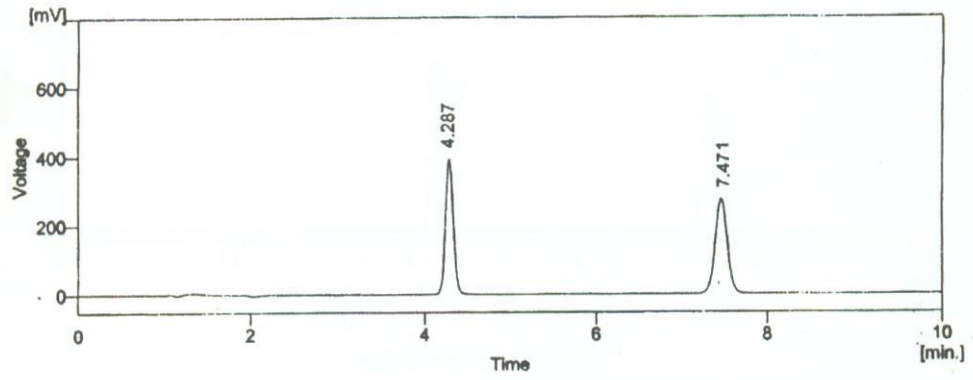
QUALITY CONTROL DEPARTMENT

SAMPLE NAME :ACCURACY -120%

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

S.NO	Reten Time (Min)	Area (mV)	Area (%)
1	4.287	2466.999	51.5
2	7.471	2251.240	48.5
	Total	4638.950	100.0

CHROMATOGRAM-30

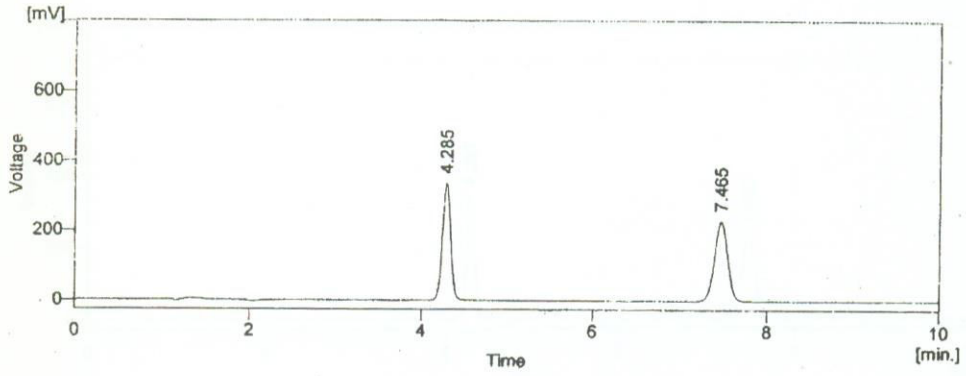
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : SYSTEM SUITABILITY 1

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result table (MET+SIT)

	Reten Time (Min)	Area (mV)	Area (%)
1	4.285	2098.834	48.6
2	7.465	2218.085	51.4
	Total	4316.920	100.0

Column Performance Table (MET+SIT)

	Reten Time (Min)	Asymmetry (-)	Efficiency (th.pl)	Resolution (-)
1	4.285	1.056	9226	-
2	7.465	1.000	11340	13.861

CHROMATOGRAM-31

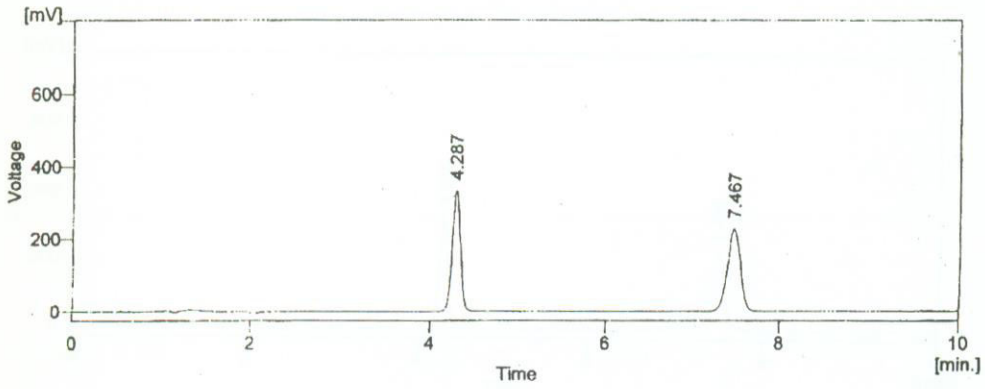
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : SYSTEM SUITABILITY 2

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result table (MET+SIT)

	Reten Time (Min)	Area (mV)	Area (%)
1	4.287	2124.436	48.9
2	7.467	2228.224	51.2
	Total	4352.660	100.0

Column Performance Table (MET+SIT)

	Reten Time (Min)	Asymmetry (-)	Efficiency (th.pl)	Resolution (-)
1	4.287	1.000	8947	-
2	7.467	1.000	12065	14.034

CHROMATOGRAM-32

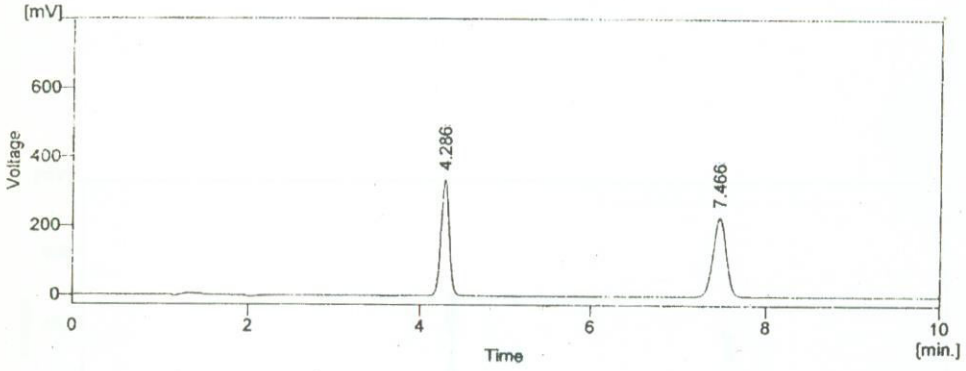
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : SYSTEM SUITABILITY 3

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result table (MET+SIT)

	Reten Time (Min)	Area (mV)	Area (%)
1	4.286	2096.984	48.4
2	7.466	2230.726	51.6
	Total	4327.71	100.0

Column Performance Table (MET+SIT)

	Reten Time (Min)	Asymmetry (-)	Efficiency (th.pl)	Resolution (-)
1	4.286	1.072	8947	-
2	7.466	1.014	12055	14.024

CHROMATOGRAM-33

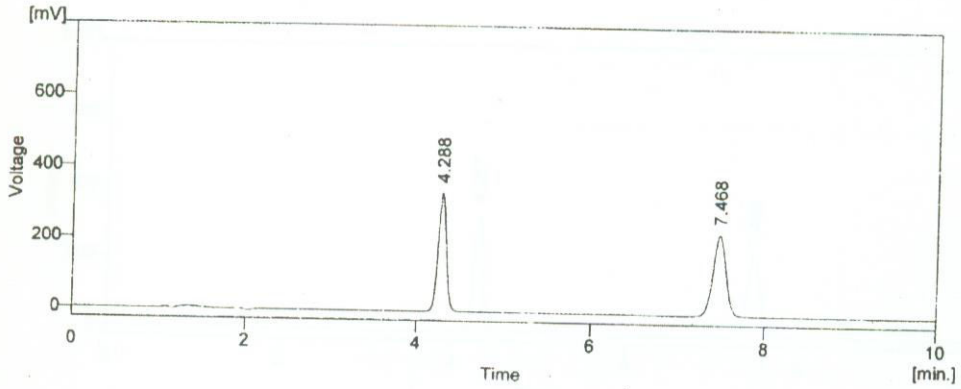
QUALITY CONTROL DEPARTMENT

SAMPLE NAME : ASSAY-1

SYSTEM : HPLC

DETECTOR : UV-VIS

TYPE OF ANALYSIS : PERCENT ON AREA



Result Table (MET+SIT)

	Reten Time (Min)	Area (mV)	Area (%)
1	4.288	2235.098	52.2
2	7.468	2051.294	47.8
	Total	4286.392	100.0

RESULT AND DISCUSSION

The working condition for the RP-HPLC method was established for Metformin HCL and Sitagliptin then was applied on pharmaceutical dosage forms. A simple reverse phase liquid chromatographic method has been developed and subsequently validated.

The separation method was carried out by using a mobile phase consisting of 0.02M dipotassium hydrogen phosphate and acetonitrile in the ratio 55:45.the detection was carried out by using UV – Visible SPD 20 A at 240nm.The column was phenominex Gemini C18 (250×4.6mm×5µ).The flow rate was selected as 1ml/min.

The retention time of Metformin HCL and Sitagliptin was found to be 4.285 and 7.485 respectively. The asymmetry factor or tailing 1.008 and 1.011respectively, which indicates symmetrical nature of the peak. The number of theoretical plates of Metformin HCL and Sitagliptin was found to be 8840 and 12044 respectively, which indicates the efficiency performance of the column.

From the linearity studies, specified concentration levels were determined .It was observed that Metformin HCL and Sitagliptin was linear in the range of 80% to 120% for the target concentration .The linearity range of 10-50mg/ml for Metformin HCL and Sitagliptin were found to obey linearity with a correlation coefficient of 0.999 and 0.999 respectively.

The validation of proposed method was verified by recovery studies. The percentage recovery range was found to be satisfied which represent in results. The robustness studies were performed by changing the pH and wavelength. The ruggedness study was also performed.

The analytical method validation was carried as per ICH guidelines and given below are the tables are the summary of the result.

S.No	PARAMETERS	LIMIT	OBSERVATIONS	PASSES/ FAILS
1	Specificity	No Interferences at retention time of the analyte peak.	No Interference at retention time of the analyte peak	Passes
2	System Precision	RSD NMT 2.0%	Metforminn:0.3397% Sitagliptin:0.385	Passes
3	Method Precision	RSD NMD 2.0%	Metforminn:0.46% Sitagliptin:0.25%	Passes
4	Linearity of detector response	Correlation co-efficient NLT 0.999	Metforminn:0.999 Sitagliptin:0.999	Passes
5	Accuracy	% Recovery range 98-102%	Metforminn:99.59-100.71% Sitagliptin:99.11-101.18%	Passes
6	Ruggedness	% Recovery range 98-102%	Within limits	Passes
7	Robustness	RSD NMT 2.0%	Within limits	Passes
8	Limit of detection (LOD)	Based on SD of the Response and slope	Metforminn :1.052µg/ml Sitagliptin:7.10µg/ml	Passes
9	Limit quantitation (LOQ)	Based on SD of the Response and slope	Metforminn :5.7µg/ml Sitagliptin 3.4µg/ml	passes

SUMMARY AND CONCLUSION

RP-HPLC method was developed. It was validated for the estimation of Metformin HCL and Sitagliptin in tablet dosage form using HPLC Shimadzu Prominence with UV-Visible SPD 20A Detector and Phenominex C18 (250x4.6mm, 5µ) column, injection of 20 µl is injected and eluted with the mobile phase of dipotassium hydrogen phosphate buffer, and acetonitrile in the ratio 55:45, which was pumped at a flow rate of 1ml at 260 nm. The peak of Metformin HCL and Sitagliptin are found well separated at 4.285 and 7.485 respectively. The developed method was validated for various parameters as per ICH guidelines like Accuracy, Precision, Linearity, Specificity, Ruggedness, Robustness, LOQ and LOD.

The analytical method validation of Metformin HCL and Sitagliptin by RP HPLC method was found to be satisfactory and could be used for the routine pharmaceutical analysis of Metformin HCL and Sitagliptin .

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